THE ROLE OF TLR2 AND TLR4 IN THE IMMUNE FUNCTIONS OF EPIDERMAL CELLS AND IN THE PATHOGENESIS OF ACNE

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Ph. D. dissertation

2006.

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Publications directly related to the subject of the dissertation

I. Kis K, Bodai L, Polyanka H, Eder K, Pivarcsi A, Duda E, Soos G, Bata-Csorgo Z, Kemeny L. Budesonide, but not tacrolimus, affects the immune functions of normal human keratinocytes. Int Immunopharmacol. 2006 Mar;6(3):358-68. IF_{2004}: 1.827

II. Pivarcsi A, Nagy I, Koreck A, Kis K, Kenderessy-Szabo A, Szell M, Dobozy A, Kemeny L. Microbial compounds induce the expression of pro-inflammatory cytokines, chemokines and human beta-defensin-2 in vaginal epithelial cells. Microbes Infect. 2005 Jul;7(9-10):1117-27. IF_{2004}: 3.753

III. Koreck A*, Kis K*, Szegedi K, Paunescu V, Cioaca R, Olariu R, Kemeny L, Dobozy A, Szell M. TLR2 and TLR4 polymorphisms are not associated with acne vulgaris. Dermatology. 2006. In press. IF_{2004}: 1.619

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Publications indirectly related to the subject of the dissertation

István Nagy, Andor Pivarcsi, Kornélia Kis, Andrea Koreck, László Bodai, Andrew McDowell, Holger Seltmann, Sheila Patrick, Christos C. Zouboulis, Lajos Kemény. Propionibacterium acnes and LPS induce the expression of antibacterial peptides and proinflammatory cytokines/chemokines in human sebocytes. Microbes Infect. 2006. In press. IF_{2004}: 3.753

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List of abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
C. albicans	*Candida albicans*
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FKBP	FK506 (tacrolimus) binding protein
GC	glucocorticoid
GR	glucocorticoid receptor
hBD2	human beta-defensin 2
IFN	interferon
IL-1	interleukin-1
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
LRR	leucine-rich repeat
mAb	monoclonal antibody
MFI	mean fluorescence intensity
NF-κB	nuclear factor kappa-B
NFAT	nuclear factor of activated T lymphocytes
P. acnes	*Propionibacterium acnes*
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PGN	peptidoglycane
RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of mean
TGF	transforming growth factor
TIR domain	Toll-interleukin-1 receptor domain
TLR	Toll-like receptor
TNF	tumor-necrosis factor

1. Introduction

Toll-like receptors (TLRs) are essential in the host defense against microbial pathogens. TLRs have emerged as the key sensors of microbial products, as they are expressed on sentinel cells in the immune system, most notably on dendritic cells and macrophages, and on epidermal cells as well, where they sense a range of chemicals produced by bacteria, viruses, fungi and protozoa [1, 2, 3]. To date 11 members of the human TLR family have been identified (TLR1-TLR11). The interaction between a TLR and a microbial component triggers the activation of the innate immune system, as well as the development of acquired immunity. Bacterial or mycobacterial lipopeptides, lipoteichoic acid, the yeast cell-wall component zymosan, or glycerophosphatidylinositol anchors from parasites are recognized by TLR2, while bacterial lipopolysaccharide (LPS) is recognized by TLR4. Structurally, TLR family members are characterized by the presence of an extracellular domain consisting of 19-25 tandem copies of leucine-rich repeat (LRR) motif and a highly conserved intracellular Toll-interleukin-1 receptor (TIR) domain. While the extracellular part is responsible for specific ligand-recognition, the intracellular part mediates the signal transduction. Recognition of pathogen-associated molecular patterns (PAMPs) by TLRs, either alone or in heterodimerization with other TLRs (TLR2/TLR1 or TLR2/TLR6) or non-TLR type coreceptors (TLR4/CD14/MD2), culminates in the activation of the transcription factor NF-κB and the MAP kinases resulting in the activation of genes important for an effective host defense, especially those of proinflammatory cytokines (Fig. 1.) [2, 4]. Keratinocytes and vaginal epithelial cells, which have been shown to express several TLRs [3, 5], have a considerable role in the initiation of host defense against infections through producing various kinds of cytokines, chemokines and antibacterial peptides. Participating in innate immune processes, epidermal and epithelial cells can contribute to the pathogenesis of numerous skin diseases, such as acne. Concerning the functions of TLR2 and TLR4 in epidermal and epithelial tissues we have proposed to investigate the following topics:

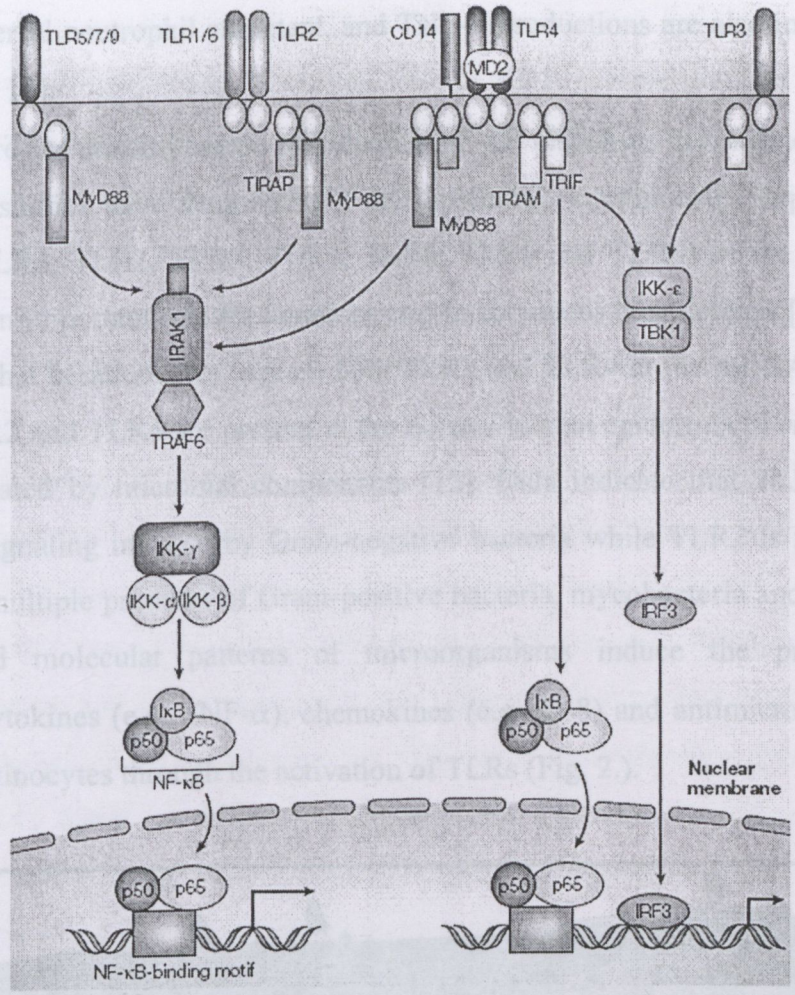


Fig. 1. Toll-like receptor signalling [4].

1.1 Effect of immunomodulators on the immune functions of keratinocytes

The skin represents the largest organ in the body, providing the principal physical barrier to the environment. Keratinocytes comprise 90% of the cells found in the epidermis and are capable of eliciting an immune response. They respond to nonspecific external stimuli with the production of inflammatory cytokines, adhesion molecules and chemotactic factors. Interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) have been called primary cytokines, activate several cellular signaling pathways, including the nuclear factor-κB (NF-κB) pathway that regulates the genes for chemokines, cytokines, defensins, E-selectin, intercellular adhesion molecule 1 and vascular-cell adhesion molecule 1 among others [3, 6-9]. IL-1 is present in keratinocytes constitutively, but upregulation has been observed upon stimulation with lipopolysaccharide (LPS), physical, chemical or thermal injury, ultraviolet irradiation or a variety of cytokines (i.e. GM-CSF, TNF-α, IL-6, TGF-α, and IL-1α and IL-1β

itself). IL-8, a powerful neutrophil attractant, and TNF-α productions are also increased after various stimuli [10, 11].

Cytokines are not the only means of inducing NF-κB responses in keratinocytes. In the past several years studies have demonstrated that epidermal keratinocytes express at least seven members (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR9) of the human TLR family suggesting an important role for keratinocytes in cutaneous host defense [3]. Recently, it has been shown that keratinocytes express both TLR2 and TLR4 at the mRNA and protein levels *in vitro*; TLR2 and TLR4 are present in the normal human epidermis *in vivo*; and their expression is regulated by microbial components [12]. Data indicate that TLR4 primarily mediates cellular signaling induced by Gram-negative bacteria while TLR2 is implicated in the recognition of multiple products of Gram-positive bacteria, mycobacteria and yeast [1, 3]. Pathogen-associated molecular patterns of microorganisms induce the production of proinflammatory cytokines (e.g. TNF-α), chemokines (e.g. IL-8) and antimicrobial peptides (e.g. hBD2) in keratinocytes through the activation of TLRs (Fig. 2.).

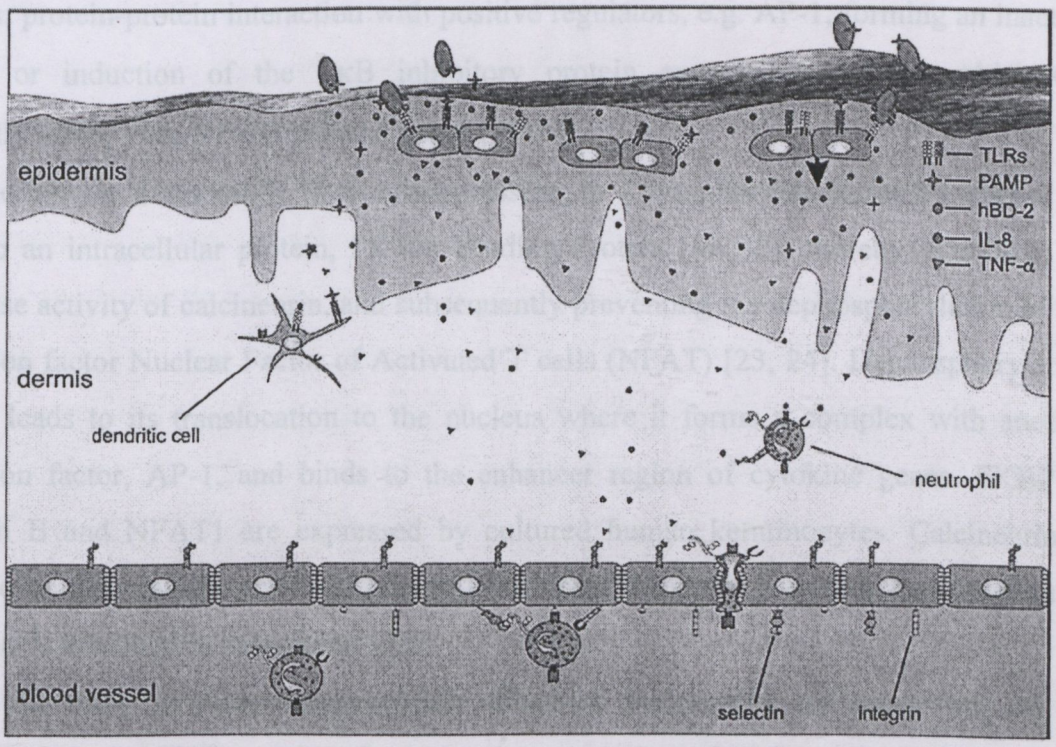


Fig. 2. Multiple roles of keratinocytes in the cutaneous immune response against infection [3].

The HaCaT keratinocyte cell line is a spontaneously transformed human epithelial cell line derived from a histologically normal skin specimen of the distant periphery of a melanoma, which maintains full epidermal differentiation capacity [13]. Despite the altered

and unlimited growth potential, HaCaT cells, similarly to normal keratinocytes, form an orderly structured and differentiated epidermal tissue, when transplanted onto nude mice. Differentiation-specific keratins (nos. 1 and 10) and other markers (involucrin and filaggrin) are expressed and regularly located. The non-tumorigenic HaCaT cells are commonly used for gene transfection studies or as a model system to study hyperproliferative skin diseases such as psoriasis, or to study cancer development [14, 15]. Cultured HaCaT cells are also frequently employed for studies of epidermal immune functions. [16-18]

Drugs, which are used in the topical treatment of inflammatory skin diseases such as psoriasis or atopic dermatitis, have a relevant influence on the immune functions of keratinocytes. Glucocorticoids are well-known anti-inflammatory agents, while tacrolimus (FK506) is the first of a new class of non-steroidal topical immunomodulators.

Glucocorticoids interact with the intracellular GC receptor (GR), which causes activation of the receptor and its translocation to the nucleus, where it regulates the expression of several genes. Induction or suppression by GR may occur through several different mechanisms, such as direct binding to GC responsive elements in GC-sensitive gene promoters; protein-protein interaction with positive regulators, e.g. AP-1, forming an inactive complex; or induction of the I-κB inhibitory protein expression, through which GR functionally inhibits the NF-κB induced genes [19-22].

Tacrolimus, a macrolide immunosuppressant, in T lymphocytes forms a complex by binding to an intracellular protein, FK506 Binding Protein (FKBP), thereby inhibiting the phosphatase activity of calcineurin, and subsequently preventing the dephosphorylation of the transcription factor Nuclear Factor of Activated T cells (NFAT) [23, 24]. Dephosphorylation of NFAT leads to its translocation to the nucleus where it forms a complex with another transcription factor, AP-1, and binds to the enhancer region of cytokine genes. FKBP12, calcineurin B and NFAT1 are expressed by cultured human keratinocytes. Calcineurin is revealed to be functionally active in human keratinocytes inducing nuclear translocation of NFAT1 that is inhibited by tacrolimus [25].

In our study we have compared the effects of budesonide and tacrolimus on the production of IL-1α, TNF-α and IL-8 and on the expression of TLR2 and TLR4 in normal human isolated keratinocytes and in HaCaT cells. Cells were co-incubated with these agents for various times. The effects of the immunosuppressants were compared on unstimulated isolated keratinocytes and on isolated keratinocytes after LPS stimulation, used as a model of

bacterial infection. We have also investigated the influence of budesonide and tacrolimus on the NF-κB activation after LPS and TNF-α induction in transfected HaCaT keratinocytes.

1.2 Effect of microbial agents on the immune functions of vaginal epithelial cells

The mucosal surface of the lower female genital tract not only provides a barrier against the outside world, but also participates in innate immune defense. Epithelial cells represent the predominant cell types that initially come into contact with microbial pathogens and play a role in immune cell recruitment and immunoregulation. This hypothesis is supported by the results of several authors. *Candida albicans* and *Streptococcus aureus* strains and superantigens were shown to upregulate the expression of chemokine and cytokine genes in reconstituted human vaginal epithelium and in human vaginal epithelial cell line [16, 27]. Besides, TLR2 and TLR4 mRNAs and proteins were detected in human female reproductive tract [28]. Immortalized human vaginal epithelial cell line was shown to express characteristic markers for basal epithelial cells, K5 and K14, and a characteristic marker for suprabasal nonkeratinizing squamous epithelium K13 [29]. These cells are also capable to secrete various soluble immunological mediators under basal culture conditions and after stimulation with exogenous cytokines [30]. Therefore this cell line provides a suitable model system to study the immunological functions of vaginal cells.

We have found that TLR2 and TLR4 receptors are expressed in vivo in the vaginal epithelia and in vitro in PK E6/E7 vaginal epithelial cell line. The mRNA expression of IL-8 and TNF-α was significantly upregulated in PK E6/E7 cells after treatment with LPS, a Gram-positive bacterial cell wall component peptidoglycan (PGN), zymosan or heat-killed *Candida albicans* (*C. albicans*). LPS, PGN and *C. albicans* also increased the mRNA level of human β-defensin 2 (hBD2), however only *C. albicans* treatment enhanced significantly the IL-1α mRNA expression. The *Mycobacterium tuberculosis* cell wall extract tuberculin had no effect on the investigated genes. The changes in hBD2 mRNA level were confirmed at the protein level as well by immunocytochemistry. We also investigated the effect of the microbial agents above on the protein expression of IL-8, IL-1α and TNF-α of the cells by ELISA to confirm our mRNA expression results.

1.3 Examination of TLR2 and TLR4 polymorphisms in acne

Acne vulgaris is a chronic inflammatory disease of the pilosebaceous unit affecting most of the teenagers and several adults. For most patients, acne is limited to occasional flares of comedones and pustules, however in severe cases, host inflammatory response can result in painful nodules and disfiguring scars. Acne is a multifactorial disease, traditional etiologic factors include increased sebum production, ductal hyperkeratosis, colonization with *Propionibacterium acnes* (*P. acnes*) and production of inflammatory mediators, but, according to twin studies, genetic factors also contribute to the development of the symptoms [31-34].

In the case of multifactorial diseases the presence of several predisposing gene alterations assists the environmental factors in eliciting the symptoms. Up to now only a few suspected genes have been investigated in connection with acne susceptibility [35-38]. Toll-like receptors (TLRs) are expressed on the surface of several immune cells and they are responsible for the recognition of microbial molecular patterns. Since TLR2 and TLR4 affect immune processes, they may play a role in the pathogenesis of acne. TLR2 and TLR4 expression was found to be increased in the epidermis of acne lesions in vivo [39], furthermore, TLR2 was expressed on the cell surface of macrophages surrounding pilosebaceous follicles and *P. acnes* induced the cytokine production of monocytes through a TLR2-dependent pathway [40].

Here we have investigated two mutations of the TLR2 gene (C2179T in the NM_003264.2 sequence and rs5743708 causing the aminoacid changes Arg677Trp and Arg753Gln, respectively) as well as two polymorphisms of the TLR4 gene (rs4986790 and rs4986791 causing the aminoacid changes Asp299Gly and Thr399Ile, respectively) in 101 Caucasian subjects, 63 with acne vulgaris and 38 healthy controls. These polymorphisms have been suggested to be associated with several infectious diseases [41]. Because the two single nucleotide polymorphisms (SNPs) of the human TLR4 gene – Asp299Gly and Thr399Ile – are situated within the extracellular domain of TLR4, the immunological impact is most likely caused by a decreased recognition of ligands. The studied nonsynonymous TLR2 SNPs are within the conserved C-terminal region of human TLR2, which mediates the signal transduction (Fig. 3.).

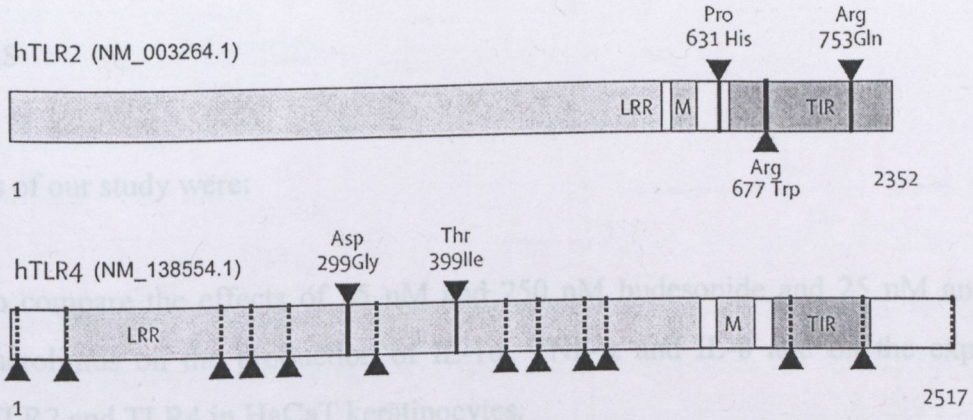


Fig. 3. Position of known SNPs within the coding sequences of TLR2 and TLR4 [41]. SNPs exhibiting allele frequencies greater than 2% are shown by full lines, frequencies less than 2% by broken lines. LRR=proposed leucine-rich repeats; M=proposed membrane-spanning region; TIR=Toll/interleukin-1 receptor domain.

2. Aims

The aims of our study were:

- to compare the effects of 25 nM and 250 nM budesonide and 25 nM and 250 nM tacrolimus on the production of IL-1α, TNF-α and IL-8 and on the expression of TLR2 and TLR4 in HaCaT keratinocytes.
- to compare the effects of 250 nM budesonide and 250 nM tacrolimus on the production of IL-1α, TNF-α and IL-8 and on the expression of TLR2 and TLR4 in isolated human keratinocytes and on the NF-κB activation after LPS and TNF-α induction in transfected HaCaT keratinocytes.
- to assess the biological relevance of TLR2 and TLR4 expression in vaginal epithelial cells by investigating the effect of microbial agents on IL-8, IL-1α and TNF-α protein expression in these cells.
- to investigate the association of two polymorphisms of the TLR2 gene and two polymorphisms of the TLR4 gene with acne vulgaris. These polymorphisms have been suggested to be associated with the susceptibility to several infectious diseases.

3. Materials and Methods

3.1 Materials

LPS (purified from *E. coli*; 026:B6), *Staphylococcus aureus* peptidoglycan (PGN), and zymosan (*Saccharomyces cerevisiae*) was purchased from Sigma (St Louis, MO). *Candida albicans* (0656 CBS Delft) was cultured on Sabourand agar and transferred to fresh agar 24 hours prior to the experiments. *Candida* cell suspension was prepared in Keratinocyte-SFM, the cells were killed by incubating them for 30 min at 56 °C. *Mycobacterium tuberculosis* cell wall extract was purchased from Human Ltd, (Gödöllő, Hungary). The extract contains the 19 kDa antigen of *M. tuberculosis*. Ethanol (absolute, reagent grade) was purchased from Spektrum-3D (Debrecen, Hungary). Budesonide was provided by Gedeon Richter, Ltd (Budapest, Hungary), and tacrolimus by Fujisawa Healthcare, Inc (Deerfield, IL). LPS was dissolved in PBS at the concentration of 10 mg/ml. Budesonide and tacrolimus were dissolved in 96% ethanol at concentrations of 25 μM and 1 mM, respectively. Stock solutions were further diluted with hydrocortisone free KGM-2 keratinocyte medium to the desired final concentrations. Human recombinant TNF-α was prepared as described previously [42].

3.2 Cell culture

3.2.1 HaCaT keratinocytes

Cells of the spontaneously transformed human keratinocyte cell line HaCaT (kindly provided by N.E. Fusenig, Heidelberg, Germany) were cultured in high-glucose DMEM (Invitrogen Corporation, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen Corporation), 1% 200 mM L-glutamine and 1% antibiotic/antimycotic solution (Sigma) (complete DMEM) at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. Vehicle (1 $^{v}/_{v}$% ethanol), 25 nM and 250 nM budesonide or 25 nM and 250 nM tacrolimus were diluted with complete DMEM then added to the culture. Supernatants and cells - homogenized in TRIzol reagent (Invitrogen Corporation) - were collected 3, 6, 12, 24 and 48 hours after addition of budesonide and tacrolimus.

3.2.2 Isolated keratinocytes

Human epidermal cells were obtained from healthy individuals undergoing plastic surgery after informed consent according to Institutional Review Board protocol. After removal of the subcutaneous tissue and much of the reticular dermis tissue, samples were cut into small strips and incubated in Dispase solution (grade II; Roche Diagnostics, Mannheim, Germany) overnight at 4°C. On the following day, the epidermis was peeled off the dermis. The epidermis was incubated in 0.25% trypsin solution (Sigma) at 37°C for 20 min and aspirated using a Pasteur pipette to aid cell dissociation. A suspension of primary epidermal cells was prepared in Keratinocyte-SFM medium (Invitrogen Corporation) supplemented with 1% 200 mM L-glutamine and 1% antibiotic/antimycotic solution (Sigma) (complete Keratinocyte-SFM medium). Epidermal cells were seeded into 75 cm^2 tissue culture flasks (Corning, Corning, NY) at a density of $7x10^4$ cells/cm^2 in complete Keratinocyte-SFM. Human epidermal keratinocytes were cultured in complete Keratinocyte-SFM in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. In our experiments, third-passage keratinocytes were used at 70-80% confluence. 24 hours before treatment the culture medium was replaced with hydrocortisone free KGM-2 keratinocyte medium (Cambrex Corporation, East Rutherford, NJ) supplemented with 1% 200 mM L-glutamine.

Vehicle (1 $^{v}/_{v}$% ethanol), 250 nM budesonide or 250 nM tacrolimus with or without 5 μg/ml LPS were diluted with hydrocortisone free KGM-2 medium then added to the culture. Supernatants and cells (homogenized in TRIzol reagent) were collected 3, 6, 12, 24 and 48 hours after addition of budesonide, tacrolimus and LPS.

3.2.3 Vaginal epithelial cells

The immortalized human vaginal epithelial cell line (PK E6/E7 cells) was a kind gift of Professor Schaeffer (North-western University Medical School, Chicago, IL) [29]. PK E6/E7 cells were cultured in complete Keratinocyte-SFM medium in a humidified atmosphere containing 5% CO_2.

PK E6/E7 cells were treated with either LPS (1 μg/ml), PGN (5 μg/ml), heat-killed *C. albicans* (10 *Candida*/PK E6/E7 cell), zymosan (10 μg/ml), tuberculin (5 μg/ml) or with microbial compound-free control medium for 0, 3, 6, 12 or 24 hours. After the indicated times, cells and supernatants were collected for further analysis.

3.3 Real-time RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen Corporation) according to the manufacturer's instructions. RNA concentration was determined by the A_{260} value of the sample. First strand cDNA was synthesized from 1.5 μg total RNA with Promega (Promega U.S., Madison, WI) ImProm-II Reverse Transcription System using a 10:1 mixture of oligo-dT and random hexamer primers. 2-4 μl of the cDNA synthesis products was used as templates in real-time PCR (iCycler IQ Real Time PCR; Bio-Rad, Hercules, CA) assays to quantify the relative mRNA levels of the genes of interest. TaqMan probes were used for TLR2, TLR4, IL-8 mRNAs and 18S rRNA while TNF-α and IL-1α mRNAs were quantitated using SYBR-Green. The following primer sets and TaqMan probes were used for RT-PCR assays: TLR2 forward: TTTCACTGCTTTCAACTGGTA, TLR2 reverse: TGGAGAGGCTGATGATGAC, TLR2 TaqMan probe: FAM/CAAGACCCACACCATCCACAA-BHQ-1/; TLR4 forward: CGATTCCATTGCTTCTTG, TLR4 reverse: GCTCAGGTCCAGGTTCTT, TLR4 TaqMan probe: FAM/CAATGCATGGAGCTGAATTTCT-BHQ-1/; IL-8 forward: CCACACTGCGCCAACA, IL-8 reverse: GCATCTTCACTGATTCTTGGAT, IL-8 TaqMan probe: FAM/CTGGGTGCAGAGGGTTGTGG-BHQ-1/; 18S RNA forward: CGGCTACCACATCCAAGGAA, 18S RNA reverse: GCTGGAATTACCGCGGCT, 18S RNA TaqMan probe: TexRed/ TGCTGGCACCAGACTTGCCCTC-BHQ-1/; TNF-α forward: TCTCCTTCCTGATCGTGGC, TNF-α reverse: GGTTCAGCCACTGGAGCT ; IL-1α forward: CAATTGTATGTGACTGCCCAAG, IL-1α reverse: ATAGTTCTTAGTGCCGTGAGTT. PCR reactions were performed in duplicates. Reaction mixtures without cDNA were used as negative control.

3.4 ELISA

IL-8, TNF-α and IL-1α protein levels in cell culture supernatants were measured using the Quantikine human IL-8, TNF-α and IL-1α immunoassay kits (R&D Systems, Minneapolis, MN) and the Biosource TNF-α EASIA kit (Biosource, Nivelles, Belgium) following the manufacturer's instructions. Color intensity was measured as the absorbance at 450 nm. According to the manufacturer, the minimum detectable concentrations were

10 pg/ml, 44.4 pg/ml and 1 pg/ml for IL-8, TNF-α and IL-1α, respectively for Quantikine kits, and 3 pg/ml for Biosource TNF-α kit.

3.5 Flow cytometry

TLR2 flow cytometry was performed using a monoclonal mouse anti-human TLR 2.1 antibody (mAb, eBioscience, San Diego, CA). 500.000 cells were washed in PBS containing BSA then incubated with TLR2 mAb conjugated with FITC for 30 min at room temperature. After washing, membrane stained cells were fixed in 1% paraformaldehyde at 4°C until analyzed. For intracellular staining, cells were fixed in 4% paraformaldehyde after membrane staining, treated with permeabilization buffer (0.1% saponine in phosphate-buffered saline containing 0.1% sodium azide and 1% FCS) and stained again with the FITC conjugated TLR2 mAb for 30 min at room temperature. After washing, membrane+intracellular stained cells were fixed in 1% paraformaldehyde and stored at 4°C until analysis. Negative controls were prepared by incubating with an isotype-matched control mouse immunoglobulin. To confirm that the staining protocol was adequate, peripheral blood lymphocytes - which strongly express TLR2 - were stained with the same method. Scanning and analysis were carried out using FACScalibur (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson). Results were expressed as the geometric mean fluorescence intensity (MFI) ratio calculated by the MFI of cells stained with an antigen-specific antibody divided by the MFI of cells stained with the isotype-matched control antibody.

3.6 NF-κB reporter assay

The effect of budesonide and tacrolimus on LPS and TNF-α induced NF-κB activation was measured using HaCaT cells stably transformed with the NF-κB/luc/neo reporter plasmid (kindly provided by Zsuzsanna Gyorfy, Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary). A neomycine resistance gene of the pRc/CMV plasmid (Invitrogen Corporation) was integrated into the original Stratagene pNF-κB/luc reporter plasmid (PathDetect® NF-κB *Cis*-Reporting System). Cells were seeded at 3×10^5 per well in a 24-well plate in DMEM:F12 1:1 medium (Invitrogen Corporation)

supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotic/antimycotic solution (Sigma) and were stimulated 12 hours later with 5 μg/ml LPS or 5 ng/ml human recombinant TNF-α. The treated cells were lysed, and the luciferase activities in lysates were measured using the Promega Luciferase Assay System according to the manufacturer's instructions (Promega).

3.7 Patients and methods for TLR genotyping

Acne vulgaris diagnosis was defined as a physician's diagnosis. Acne patients were subclassified into three groups, as having severe symptoms (acne conglobata group n=17), medium symptoms (acne papulo-pustulosa group, n=39) and mild symptoms (acne comedonica group, n=7). Control group comprised patients showing no symptoms of acne during their life.

We isolated genomic DNA from whole blood obtained from the study subjects, using the Perfect gDNA Blood Mini Isolation Kit (Eppendorf AG, Hamburg, Germany) and Generation Capture Column Kit (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Informed consent was obtained from each individual before sample collection and the study was conducted according to the Helsinki Declarations. Primer sets were designed by Primer3 software at http://frodo.wi.mit.edu/cgi-bin/primer3 such that products did not exceed 300 bp and synthesized by Oligonucleotide Laboratory, Biological Research Center of the Hungarian Academy of Sciences (Szeged, Hungary). PCR was performed in a total volume of 50 μl containing 200 ng of template DNA, 1-1 μl of 10 pmol/μl primers and 25 μl REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich Co., St. Louis, MO). The cycling profile involved denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min for 40 cycles. Final extension was continued at 72°C for 10 min. The amplification was carried out in a MyCycler Thermal System (Bio-Rad, Hercules, CA). PCR products were purified by Quantum Prep PCR Kleen Spin Columns (Bio-Rad) according to the manufacturer's instructions. The alleles of TLRs were confirmed by sequencing (Agricultural Biotechnology Center, Gödöllő, Hungary) and analysed by BioEdit program (Ibis Therapeutics, Carlsbad, CA).

To assess the effect of the studied TLR polymorphisms on the protein structure and function, an *in silico* analysis was performed. We used PolyPhen software (http://tux.embl-heidelberg.de/ramensky/polyphen.cgi) to predict the possible impact of an amino acid

substitution on the protein. This calculation is based on the observation that the studied substitution is rarely or never detected in the family of homologous proteins [43]. Changes in protein hydrophilicity resulted from the occurrence of the studied SNPs were observed at the homepage http://bioinformatics.org/JaMBW, which uses the Hopp and Wood algorithm to determine the charged areas of a protein.

3.8 Data presentation

PCR results were expressed as fold increases over the 0 hour or control values. Data were presented as mean ± standard error of mean (SEM) for *n* experiments. Flow cytometry data were expressed as the geometric mean fluorescence intensity (MFI) ratio. Data were compared using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or Mann-Whitney U test to determine statistical differences after multiple comparisons (SPSS, SPSS Inc., Chicago, IL). Probability values of less than 0.05 for parametric tests and less than 0.005 (for keratinocytes experiments) or 0.05 (for vaginal epithelial cell experiments) for nonparametric tests were considered significant.

The statistical analysis of allele frequency data was performed using the statistical homepage faculty.vassar.edu/lowry/VassarStats.html. Data were compared using Fisher exact probability test, a p value less than 0.05 was considered significant.

4. Results

4.1 Effects of immunemodulators on keratinocytes

4.1.1 TLR2 and TLR4 mRNA expressions are suppressed by tacrolimus in HaCaT keratinocytes

The effects of the immunosuppressive drugs budesonide and tacrolimus on the constitutive gene expressions of IL-8, TNF-α and IL-1α and the Toll-like receptors 2 and 4 were examined in HaCaT keratinocytes. HaCaT cells were treated with 25 nM and 250 nM budesonide or 25 nM and 250 nM tacrolimus or with solvent. Samples were collected 3, 6, 12, 24 and 48 hours after treatment and were subjected to real-time RT-PCR. IL-8 mRNA was slightly suppressed after the treatment with both concentrations of budesonide (Fig. 4B). A slight increase in TLR2 mRNA was noticed in two out of three experiments in HaCaT cells at 24 hours of coincubation with the steroid, mainly when it was applied at the higher concentration. Furthermore, 250 nM tacrolimus significantly decreased the mRNA expressions of TLR2 and TLR4, while it did not change the expressions of the other examined genes (Fig. 4D, E). Neither drugs influenced the mRNA levels of IL-1α and TNF-α.

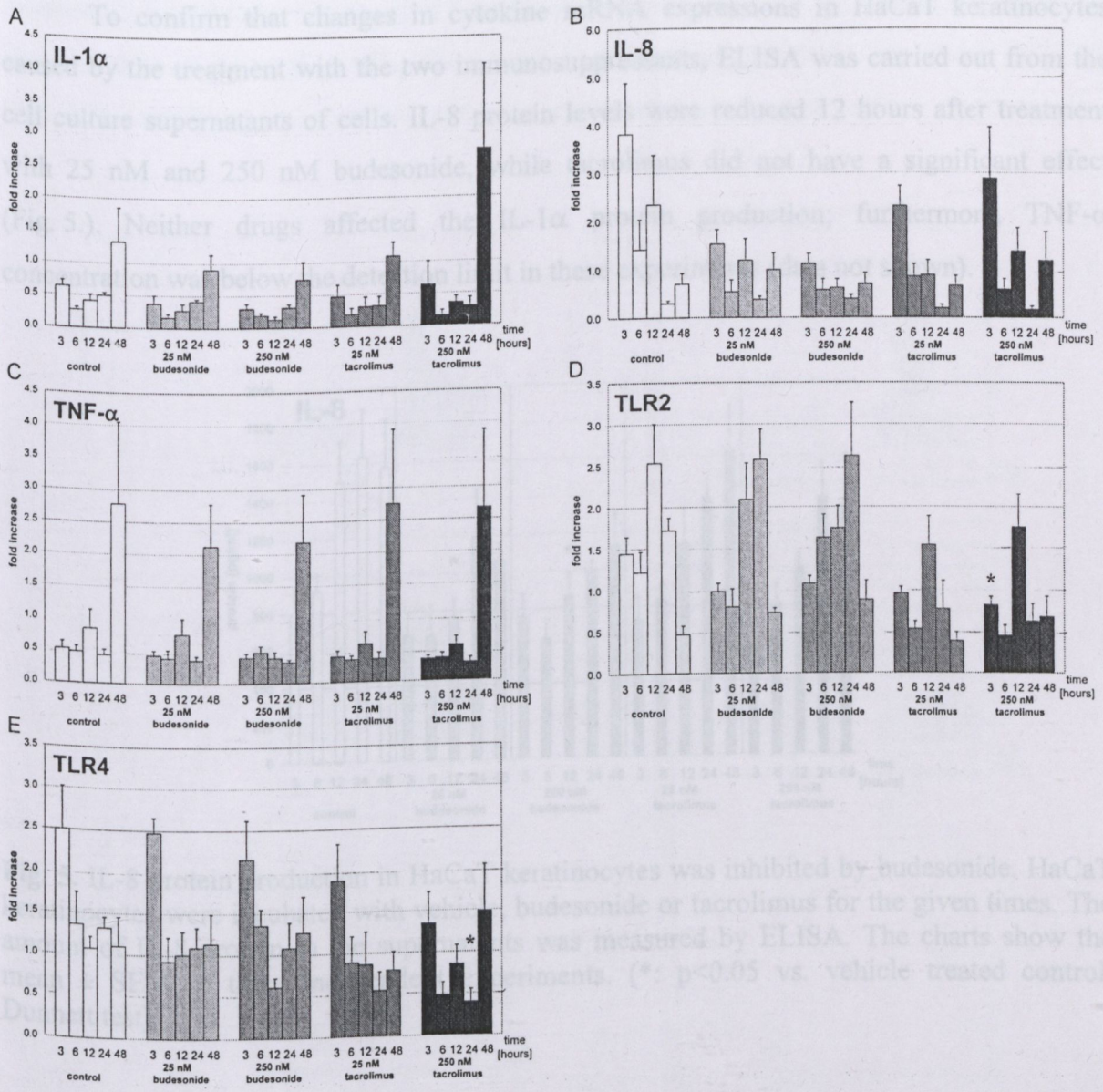


Fig. 4. Constitutive expressions of TLR2 and TLR4 mRNAs were inhibited significantly by tacrolimus. Expressions of IL-1α (A), IL-8 (B), TNF-α (C), TLR2 (D) and TLR4 (E) genes were analyzed by real-time RT-PCR. Total RNA from HaCaT keratinocytes incubated with vehicle, budesonide or tacrolimus for the given times was reverse transcribed and subjected to real-time RT-PCR. The levels of mRNAs were normalized to the 18S rRNA, and are presented as fold increases over the 0-hour values. The chart shows the mean ± SEM of four independent experiments (*: $p<0.05$ vs. vehicle treated control, Dunnett test).

4.1.2 Budesonide significantly decreased the IL-8 protein expression in HaCaT keratinocytes

To confirm that changes in cytokine mRNA expressions in HaCaT keratinocytes caused by the treatment with the two immunosuppressants, ELISA was carried out from the cell culture supernatants of cells. IL-8 protein levels were reduced 12 hours after treatment with 25 nM and 250 nM budesonide, while tacrolimus did not have a significant effect (Fig. 5.). Neither drugs affected the IL-1α protein production; furthermore, TNF-α concentration was below the detection limit in these experiments (data not shown).

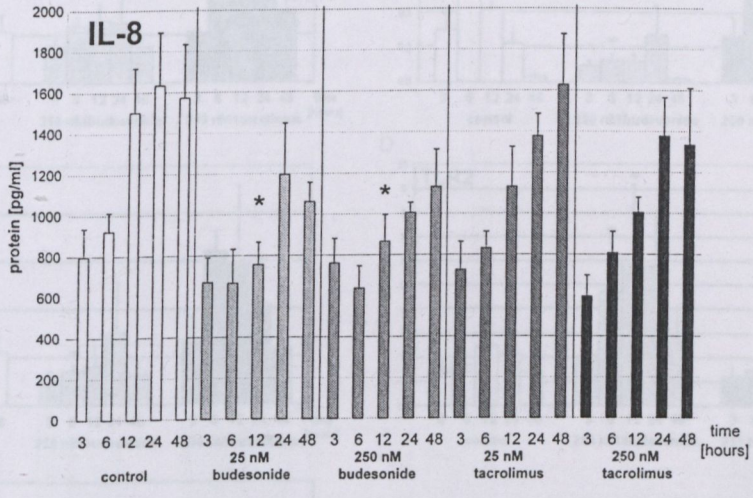


Fig. 5. IL-8 protein production in HaCaT keratinocytes was inhibited by budesonide. HaCaT keratinocytes were incubated with vehicle, budesonide or tacrolimus for the given times. The amount of IL-8 protein in the supernatants was measured by ELISA. The charts show the mean ± SEM of three independent experiments. (*: $p<0.05$ vs. vehicle treated control, Dunnett test).

4.1.3 IL-8 and TNF-α mRNA expressions are suppressed, TLR2 mRNA expression is induced by budesonide, but not by tacrolimus, in cultured human keratinocytes

In order to examine the effect of the budesonide and tacrolimus on the constitutive expression of pro-inflammatory cytokines IL-8, TNF-α and IL-1α and the TLR2 and TLR4, subconfluent cultures of keratinocytes were incubated with 250 nM budesonide or 250 nM tacrolimus and subjected to real-time RT-PCR. Samples were collected 3, 6, 12, 24 and 48 hours after addition of drugs, vehicle treated samples were used as controls. The amount of IL-8 and TNF-α mRNA were decreased significantly and a moderate fall in case of IL-1α mRNA was noticeable at 6 hours after co-incubation with 250 nM budesonide (Fig. 6A, B, C). At the subsequent time points the values returned to control values. TLR2 expression has

increased 5-6 fold following the first 3 hours of co-incubation with the steroid and remained elevated up to 24 hours (Fig. 6D). There were no changes in the mRNA expression of TLR4 (Fig. 6E). Tacrolimus had no effect on the expression of the examined genes.

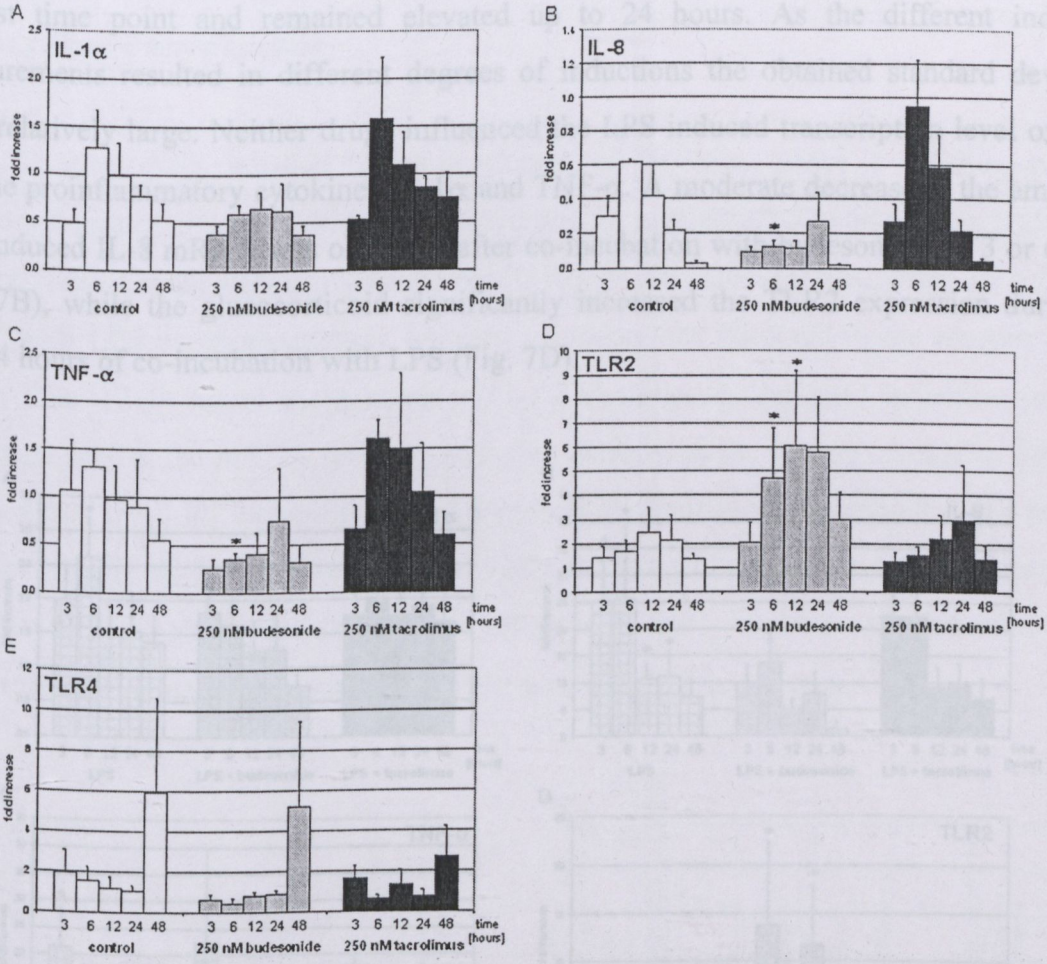


Fig. 6. Constitutive expressions of IL-8, TNF-α and TLR2 mRNAs were influenced by budesonide, but not by tacrolimus. Expressions of IL-1α (A), IL-8 (B), TNF-α (C), TLR2 (D) and TLR4 (E) genes were analyzed by real-time RT-PCR. Total RNA from third passage isolated keratinocytes incubated with vehicle, budesonide or tacrolimus for the given times was reverse transcribed and subjected to real-time RT-PCR. The levels of mRNAs were normalized to the 18S rRNA, and are presented as fold increases over the 0-hour values. The chart shows the mean ± SEM of four independent experiments (*: $p<0.05$ vs. vehicle treated control, Dunnett test).

4.1.4 Budesonide slightly hindered the LPS induced increase of IL-8 expression but elevated the mRNA level of TLR2

To investigate whether the drugs are able to suppress LPS induced immune responses in keratinocytes, cells were stimulated with 5 μg/ml LPS in the presence of 250 nM

budesonide, 250 nM tacrolimus or solvent. Samples were collected 3, 6, 12, 24 and 48 hours after treatment and subjected to real-time RT-PCR analyses. LPS significantly induced the expression of IL-1α, IL-8 and TNF-α, and slightly increased the amount of TLR4 mRNA (Fig. 7A, B, C, E). Induction of IL-8 and TNF-α was pronounced, about 20-25 fold at the earliest time point and remained elevated up to 24 hours. As the different individual measurements resulted in different degrees of inductions the obtained standard deviations were relatively large. Neither drugs influenced the LPS induced transcription level of TLR4 and the proinflammatory cytokines IL-1α and TNF-α. A moderate decrease in the amount of LPS induced IL-8 mRNA was observed after co-incubation with budesonide for 3 or 6 hours (Fig. 7B), while the glucocorticoid significantly increased the TLR2 expression during the first 24 hours of co-incubation with LPS (Fig. 7D).

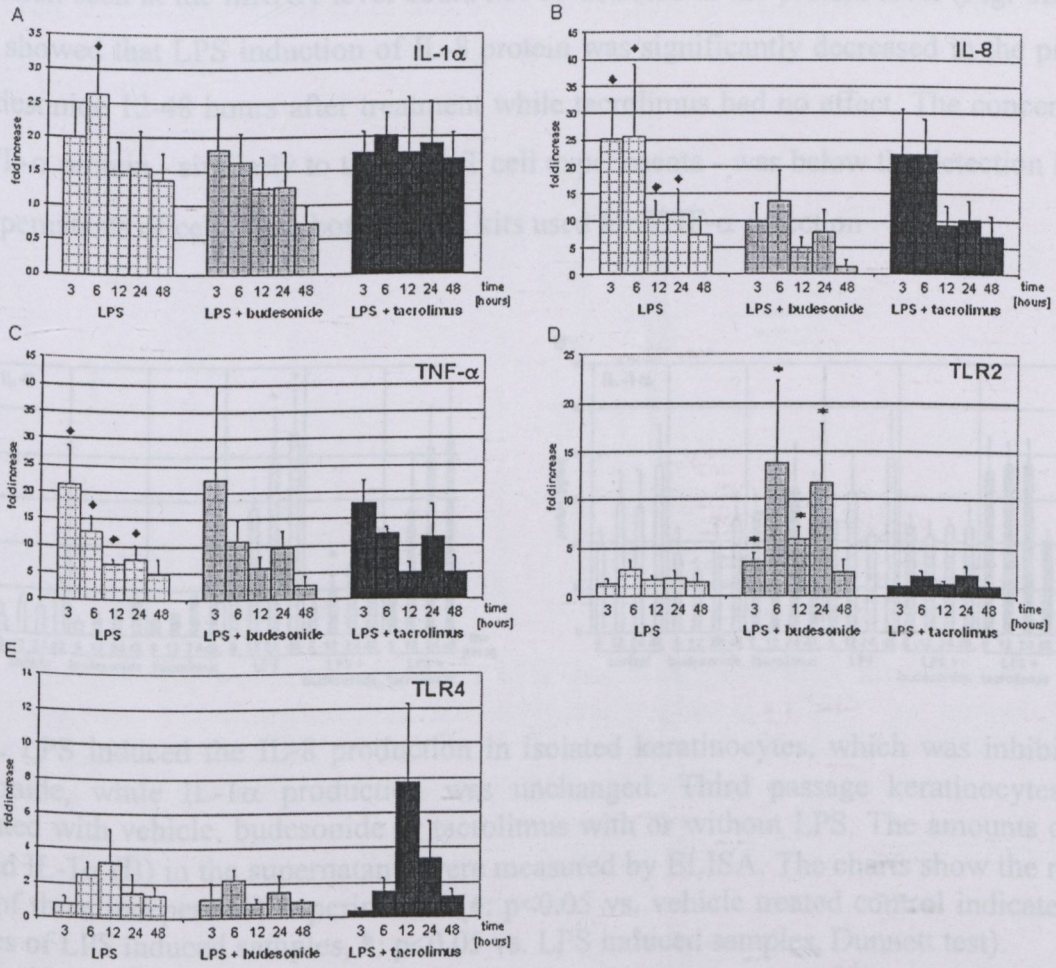


Fig. 7. LPS induced IL-1α, IL-8, and TNF-α mRNA expressions, budesonide slightly inhibited this increase in the case of IL-8 expression, while increased the TLR2 expression. Total RNA from third passage isolated keratinocytes incubated with vehicle, budesonide or tacrolimus in the presence of LPS for the given times was reverse transcribed and subjected to real-time RT-PCR. The levels of mRNAs were normalized to the 18S rRNA, and are presented as fold increases over the vehicle treated samples, respectively. The chart shows the

mean ± SEM of three independent experiments (♦ : $p<0.05$ vs. vehicle treated control indicated only in cases of LPS induced samples, *: $p<0.05$ vs. LPS induced samples, Dunnett test).

4.1.5 Suppressive effect of budesonide on IL-8 expression was also revealed at the protein level in cultured keratinocytes

To determine whether down-regulation of cytokine genes is accompanied by diminished protein levels, ELISA was carried out. In unstimulated keratinocytes budesonide and tacrolimus did not alter significantly the IL-8 or IL-1α protein levels. When the cells were treated with LPS an approximately fourfold induction was observed in the amount of IL-8 protein after 12-48 hours of incubation (Fig. 8A), however the mild induction in IL-1α expression seen at the mRNA level could not be detected at the protein level (Fig. 8B). IL-8 assay showed that LPS induction of IL-8 protein was significantly decreased in the presence of budesonide 12-48 hours after treatment while tacrolimus had no effect. The concentration of TNF-α protein - similarly to the HaCaT cell experiments - was below the detection limit in the supernatants of cells with both ELISA kits used for TNF-α detection

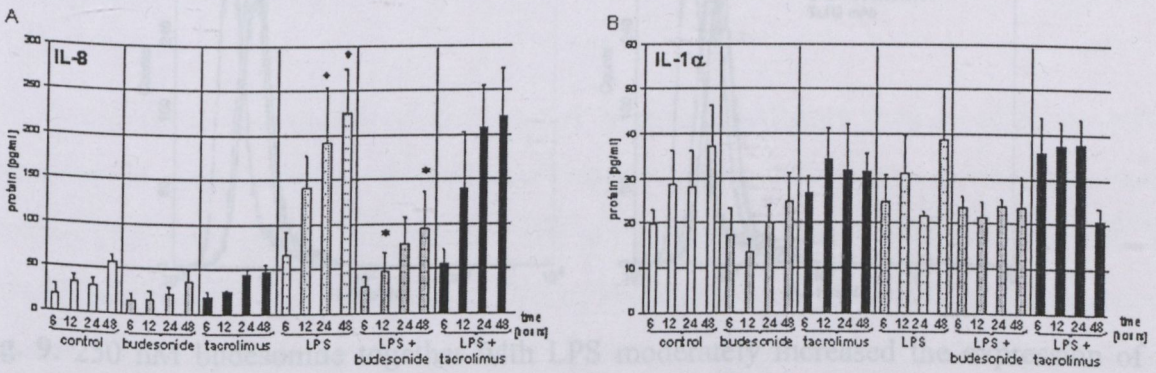


Fig. 8. LPS induced the IL-8 production in isolated keratinocytes, which was inhibited by budesonide, while IL-1α production was unchanged. Third passage keratinocytes were incubated with vehicle, budesonide or tacrolimus with or without LPS. The amounts of IL-8 (A) and IL-1α (B) in the supernatants were measured by ELISA. The charts show the mean ± SEM of three independent experiments. (♦: $p<0.05$ vs. vehicle treated control indicated only in cases of LPS induced samples, *: $p<0.05$ vs. LPS induced samples, Dunnett test).

4.1.6 A moderate increase in the level of TLR2 protein was observed in LPS stimulated cells after co-incubation with budesonide

36 hours after incubation of normal human keratinocytes with budesonide, tacrolimus or solvent in the presence or absence of LPS, cells were trypsinized and stained with FITC conjugated TLR2 mAb. Both membrane and membrane+intracellular staining procedures were performed. Staining of cells showed no detectable amount of TLR2 on the cell membrane even after LPS induction, however a strong intracytoplasmic TLR2 expression was observed (Fig. 9., Fig. 10.). LPS treatment alone did not increase the expression of intracellular TLR2 protein, but LPS together with budesonide caused a moderate elevation (Fig. 9B). Tacrolimus did not influence the expression of TLR2 protein.

To exclude that trypsin exposure resulted in the removal of TLR2 from the cell surface peripheral blood samples were hemolyzed, treated with trypsin under the same conditions than keratinocytes then stained with FITC-conjugated anti-human TLR2 mAb and the corresponding isotype control. There was no difference between the staining of trypsin treated blood lymphocytes and untreated control samples suggesting that trypsinization did not affect the membrane expression of TLR2 (data not shown).

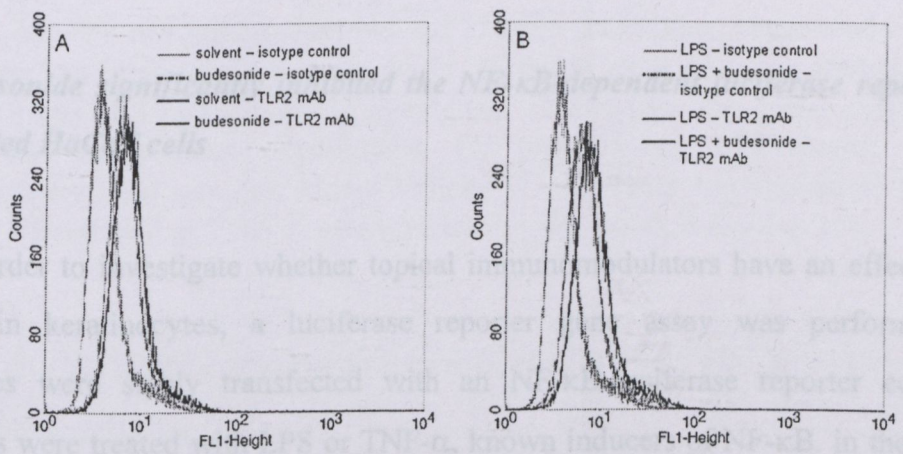


Fig. 9. 250 nM budesonide together with LPS moderately increased the expression of the intracytoplasmic TLR2 protein. Membrane+intracytoplasmic TLR2 in normal human keratinocytes was stained and analyzed by flow cytometry after incubation with solvent, budesonide (A), LPS or LPS and budesonide (B) for 36 hours.

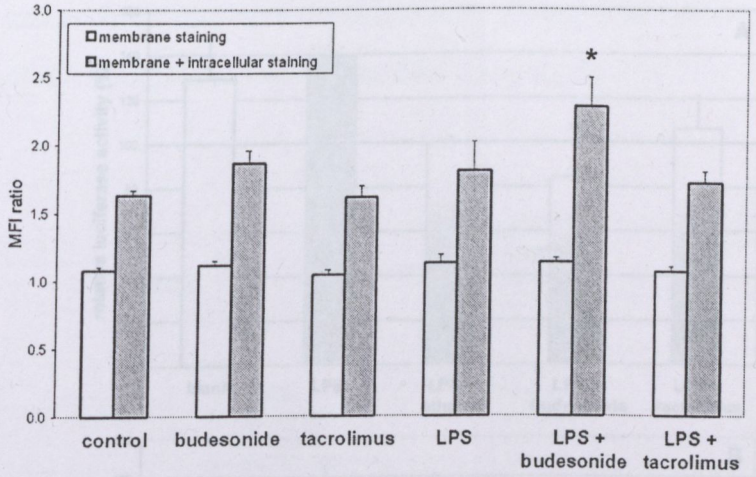


Fig. 10. MFI ratio of isolated keratinocytes stained for either membrane or membrane+intracellular detection with mouse anti-human FITC-TLR2 mAb 36 hours after treatment with solvent, 250 nM budesonide, 250 nM tacrolimus, 5 μg/ml LPS, and LPS together with budesonide or tacrolimus. The chart shows the mean ± SEM of four independent experiments for tacrolimus and LPS + tacrolimus treated samples and six independent experiments for the other samples. (*: $p<0.05$ vs. vehicle treated control, Dunnett test) MFI ratio: geometric mean fluorescence intensity (MFI) of cells stained with the antigen-specific antibody divided by the MFI of cells stained with isotype-matched control mouse immunoglobulin.

4.1.7 Budesonide significantly inhibited the NF-κB-dependent luciferase reporter activity of transfected HaCaT cells

In order to investigate whether topical immunomodulators have an effect on NF-κB activation in keratinocytes, a luciferase reporter gene assay was performed. HaCaT keratinocytes were stably transfected with an NF-κB-luciferase reporter construct and transfectants were treated with LPS or TNF-α, known inducers of NF-κB, in the presence or absence of budesonide or tacrolimus (Fig. 11.), or remained untreated for 12 hours. Results of the assays demonstrated that TNF-α induced a marked activation of NF-κB in keratinocytes, which was significantly inhibited by budesonide. LPS slightly increased the luciferase activity in keratinocytes, which was also significantly inhibited by budesonide suggesting that this drug has a suppressive effect on keratinocyte immune functions (Fig. 11.). In both LPS and TNF-α treated cells ethanol alone reduced the activity of NF-κB. In contrast to budesonide, tacrolimus had no effect on NF-κB activation in keratinocytes after LPS or TNF-α treatment.

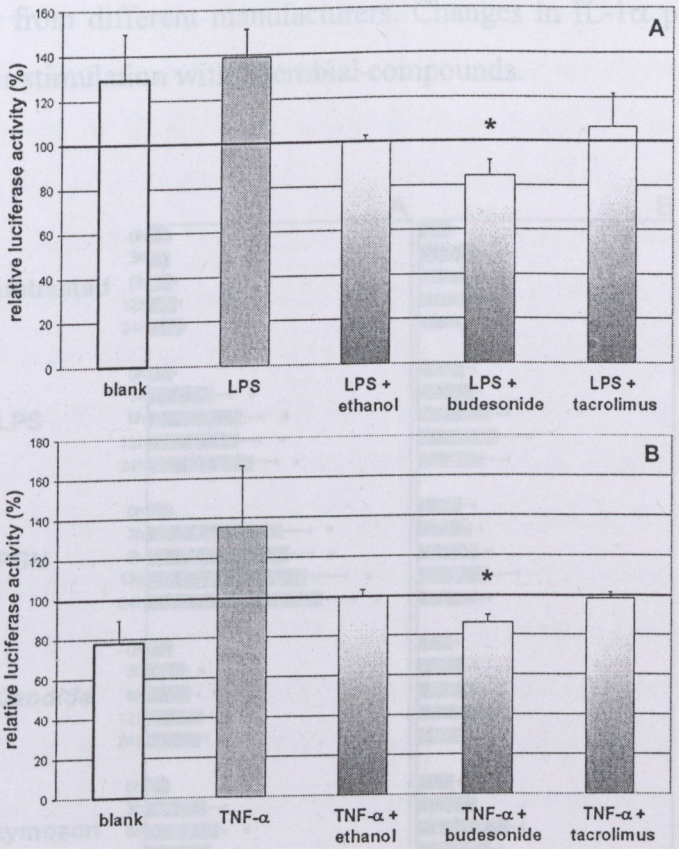


Fig. 11. 250 nM budesonide, but not 250 nM tacrolimus, significantly inhibited the NF-κB-dependent luciferase reporter activity of HaCaT cells 12 hours after induction with 5 μg/ml LPS (A) or 5 ng/ml human recombinant TNF-α (B). The charts show the mean ± SEM of four independent experiments. (*: $p<0.005$ vs. LPS+ethanol (solvent) or TNF-α+ethanol treated samples, Mann-Whitney U test) Thick line represents the luciferase activity of LPS+ethanol or TNF-α+ethanol treated cells, which is equal to 100.

4.2 Effect of microbial compounds on IL-8, IL-1α and TNF-α protein expression in vaginal epithelial cell line

To assess the biological relevance of TLR2 and TLR4 expression in vaginal epithelial cells, we investigated the effects of microbial compounds on the protein expression of IL-8, IL-1α and TNF-α. We also investigated if these changes were in agreement with our mRNA results. The IL-8 secretion of vaginal epithelial cells was significantly induced by LPS, PGN, zymosan and heat-killed *C. albicans*, while tuberculin treatment had no effect as demonstrated by ELISA (Fig. 12.). The concentration of IL-8 in the supernatant of PK E6/E7 cells constantly increased until the last time point we analyzed (24 hours). Although LPS, PGN, *C. albicans* and zymosan significantly induced the expression of TNF-α at the mRNA level, TNF-α protein was not detectable in the cell culture supernatants although we tried two

different ELISA assays from different manufacturers. Changes in IL-1α protein expression were not significant after stimulation with microbial compounds.

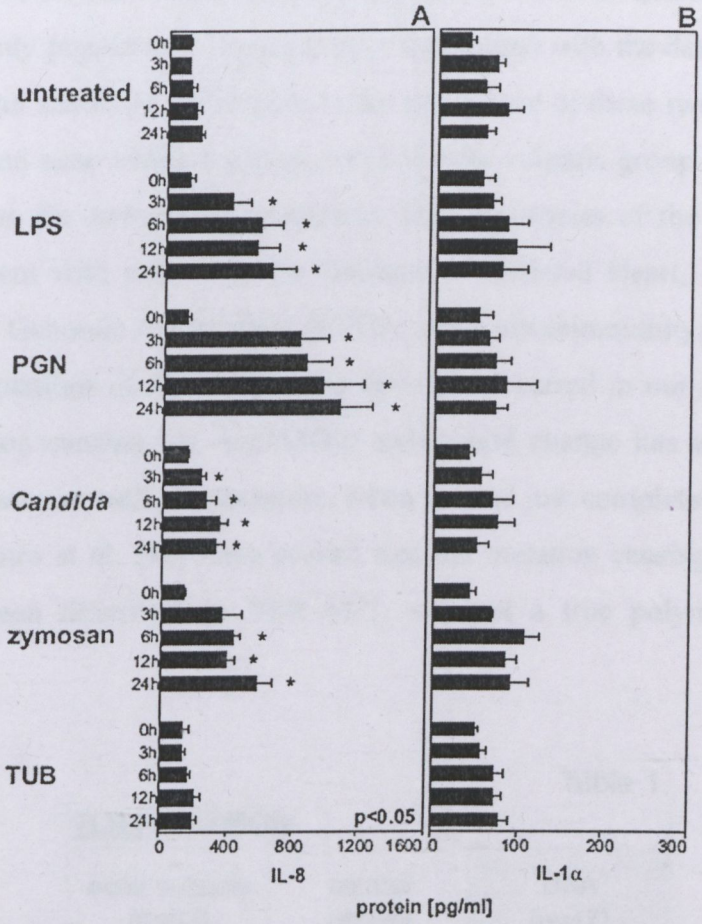


Fig. 12. Microbial compounds representing Gram-negative, Gram-positive bacteria and fungal pathogens induced IL-8 protein production in vaginal epithelial cells. Concentrations of IL-8 (A) and IL-1α (B) were measured by ELISA in the supernatants obtained from cultured immortalized vaginal epithelial cells treated with LPS, PGN, heat-killed *C. albicans*, zymosan and mycobacterial cell wall extract (TUB). Concentrations are shown as picograms of protein per ml of supernatant. Data are presented as mean ± SEM of n≥3 experiments. A statistical analysis was performed, asterisks represent significant differences compared to 0-hour values ($p<0.05$).

4.3 Examination of TLR2 and TLR4 polymorphisms in acne

4.3.1 The prevalence of the studied TLR2 and TLR4 alleles in acne patients was similar to the prevalence of these alleles found in healthy individuals.

Allele frequencies of the studied SNPs of TLR4 gene causing the amino acid changes Asp299Gly and Thr399Ile in the acne vulgaris and control group are shown in Table 1. The two studied polymorphisms of TLR4 gene occurred simultaneously (n=11) composing a haplotype. Furthermore, the individuals carrying these alleles were all heterozygotes for both TLR4 SNPs in our study population. These findings correspond with the data in the literature [44, 45]. There were no statistical differences in the prevalence of these two polymorphisms between the control and acne vulgaris groups, even if acne vulgaris group was divided into subclasses according to the severity of symptoms. The frequencies of the observed alleles were in good agreement with data from the database of National Heart, Lung and Blood Institute, Programs for Genomic Applications (IIPGA, www.innateimmunity.net).

The studied mutations of the TLR2 gene have not occurred in our study population (Table 2.). The mutation causing the Arg753Gln amino acid change has a low prevalence according to the databases as well. Furthermore, when we had just completed the sequencing of our samples, Malhotra et al. [46] have proved that the mutation causing the Arg677Trp change, which had been described in 2001 [47], was not a true polymorphism, but a sequencing error.

Table 1.

TLR4 Asp299Gly	acne vulgaris (n=63)	control (n=38)	USA* (n=47)
A:	0.952	0.934	0.904
G:	0.048	0.066	0.096

TLR4 Thr399Ile	acne vulgaris (n=72)	control (n=39)	USA* (n=47)
C:	0.958	0.936	0.947
T:	0.042	0.064	0.053

*: from the database of IIPGA, www.innateimmunity.net

Table 1. Allele frequencies of the observed polymorphisms (rs4986790 and rs4986791) in TLR4 gene causing the aminoacid changes Asp299Gly and Thr399Ile, respectively.

Table 2.

TLR2 Arg677Trp	
acne vulgaris (n=63)	control (n=38)
SNP is not detected	

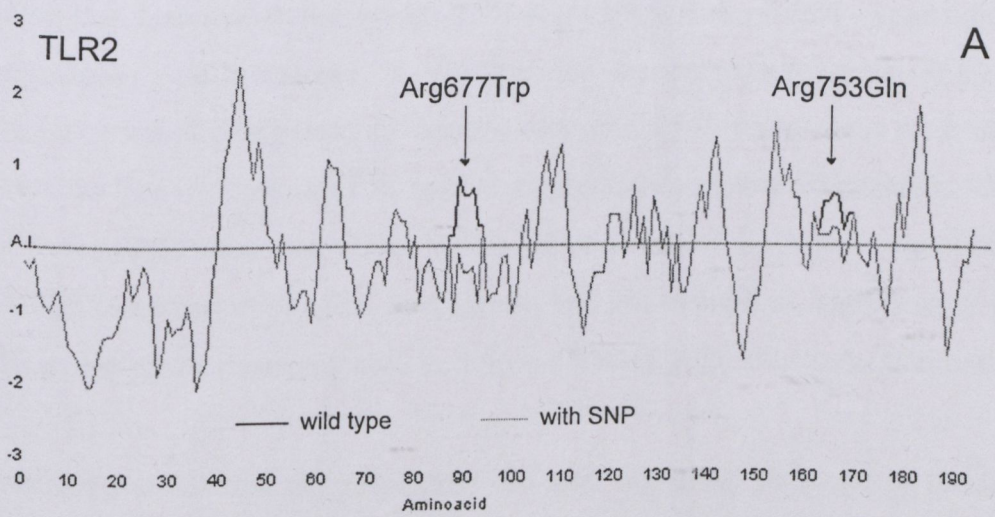
TLR2 Arg753Gln			
	acne vulgaris (n=63)	control (n=38)	USA* (n=89)
G:	SNP is not detected		0.977
A:			0.023

*: from the database of IIPGA, www.innateimmunity.net

Table 2. Allele frequencies of the observed mutations (C2179T in the NM_003264.2 sequence and rs5743708) in TLR2 gene causing the aminoacid changes Arg677Trp and Arg753Gln, respectively.

4.3.2 In silico analysis

With *in silico* analysis, TLR2 Arg677Trp and Arg753Gln amino acid changes were predicted to be probably and possibly damaging to the protein function, respectively. Furthermore, the studied amino acid changes in TLR2 protein were predicted to result in a considerable alteration in the hydrophilicity of the protein, while the studied SNPs of TLR4 didn't have a significant impact on the TLR4 protein (Fig. 13.).



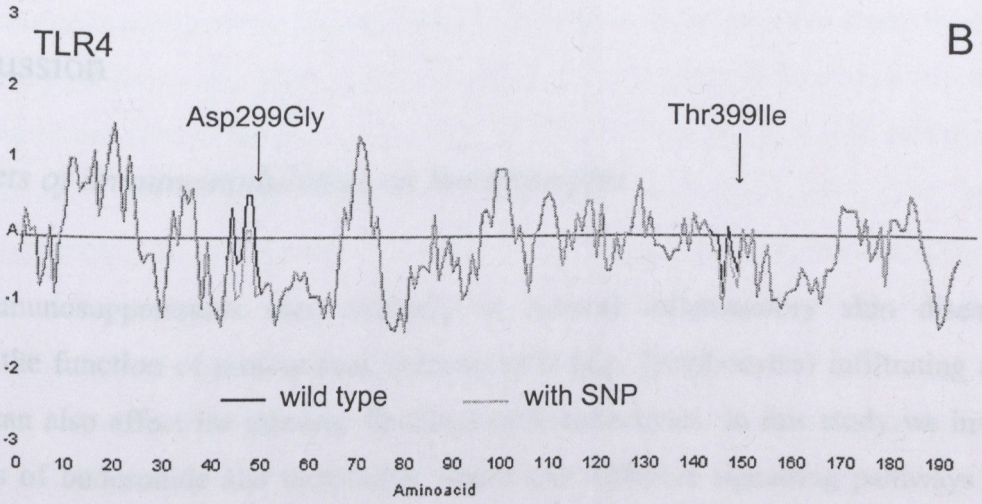


Fig. 13. Arg677Trp and Arg753Gln aminoacid changes of TLR2 protein caused a considerable alteration in the hydrophilicity of the protein (A), however the studied aminoacid changes of TLR4 didn't have a significant impact on the TLR4 protein (B) as it was seen at the http://bioinformatics.org/JaMBW homepage. The figure shows a 200-bp aminoacid sequence of the TLR2 and TLR4 protein.

5. Discussion

5.1 Effects of immunemodulators on keratinocytes

Immunosuppressants used topically in several inflammatory skin diseases can influence the function of professional immune cells (e.g. lymphocytes) infiltrating the skin, but they can also affect the immune functions of keratinocytes. In this study we investigate the effects of budesonide and tacrolimus, which use different signalling pathways to exert their immunomodulatory effects, on the IL-1α, IL-8, TNF-α production and TLR2 and TLR4 expression of keratinocytes.

We used HaCaT keratinocytes for our first experiments. These cells are considered to be good models of normal human keratinocytes, they are easily available and relatively easy to work with. The immunosuppressants were used at 25 nM and 250 nM concentrations. 250 nM tacrolimus significantly decreased the mRNA expressions of TLR2 and TLR4; furthermore, both concentrations of budesonide decreased the IL-8 mRNA and protein levels of cells. There were no significant changes in the expression of other observed genes (IL-1α and TNF-α) in HaCaT keratinocytes.

Human keratinocytes isolated from normal skin and cultured in vitro were also used to compare the effects of 250 nM of budesonide and tacrolimus on the IL-1α, IL-8, TNF-α production and TLR2 and TLR4 expression. We found that budesonide suppressed both constitutive and LPS induced IL-8 mRNA expression of keratinocytes. It was also demonstrated that budesonide suppressed LPS stimulated IL-8 protein expression. TNF-α mRNA expression was decreased in unstimulated keratinocytes, while TLR2 mRNA expression was markedly enhanced in unstimulated and LPS treated cells after incubation with budesonide. The increase in TLR2 mRNA expression was also detectable at the protein level in LPS stimulated cells. Tacrolimus had no effect on IL-1α, IL-8, TNF-α production and TLR2 and TLR4 expression of cells. Budesonide, but not tacrolimus, significantly inhibited the NF-κB-dependent luciferase reporter activity in HaCaT cells after induction with LPS or TNF-α.

Although steroids and tacrolimus use different signaling pathways to develop their effects, it is known that both can inhibit IL-1β production in LPS stimulated human monocytic cell line [48] and IL-2, IL-3, IL-4, IL-5, IFN-γ production in peripheral blood

mononuclear cells stimulated with anti-CD3/CD2 [49]. In a comparative study the effects of 10-1000 nM tacrolimus and 1000 nM dexamethasone were examined on passively sensitized human lung tissue after exposure to mite antigen. Both agents caused a significant inhibition of TNF-α and IL-5 mRNA expression and protein production [50]. The effects of immunosuppressants on inflammatory signals of keratinocytes have been examined with and without stimulation of the cells, but the results are contradictory. Michel et al. have shown that IL-8 mRNA levels were decreased in normal human keratinocytes after treatment with over 50 nM tacrolimus for 1-3 hours [51], however Kaplan et al. showed no effect on keratinocyte IL-8 protein after 1-5000 nM tacrolimus treatment for 24 hours [52]. In another experiment 10-1000 nM tacrolimus has significantly upregulated the release of transforming growth factor-β (TGF-β) in the presence of TNF-α in keratinocytes, at the same time it downregulated the mRNA and protein expression of inducible nitric oxide synthase (iNOS) [53]. It has also been demonstrated that glucocorticoids (0.1-1000 nM) by themselves could augment the production of IL-1α by PAM 212 cells (murine epithelial cell line) at both the protein and mRNA levels after 6 hours of co-incubation. When these cells were stimulated for 24 hours with haptens or irritants, which alone increased the IL-1α production, glucocorticoids synergistically enhanced the effect [54, 55]. NF-κB activation was also revealed in parallel with these observations, nevertheless Lukiw et al. showed inhibition of DNA binding of NF-κB by 100 nM budesonide epimer R and dexamethasone in keratinocytes activated by IL-1β or platelet-activating factor (PAF) [21]. The discrepancies may be due to the different experimental settings, e.g. different sampling time points, different cell types and concentrations of immunosuppressants used in the experiments.

In our experiment we applied 250 nM budesonide or tacrolimus on keratinocytes with or without stimulation with 5 μg/ml LPS, because it has been previously reported that 250 nM concentration of either tacrolimus or steroids were effective in keratinocytes and other cell types in influencing various immune functions of cells, such as DNA binding activity of transcription factors, IL-8 mRNA production, TGF-β or iNOS expression in keratinocytes; proliferation of peripheral blood T-lymphocytes; or TNF-α and IL-5 expression of passively sensitized lung tissues [21, 50, 51, 53, 56]. It is also known that LPS used in the range of 0.1-10 μg/ml can induce the IL-8 production of human keratinocytes [57]. Tacrolimus was shown to decrease the viability of normal human keratinocytes over 1000 nM concentration [52, 53]. In preliminary experiments we have checked the toxicity of the 250 nM of budesonide and tacrolimus on normal human keratinocytes and found no toxic effect (data not shown). With

the choice of 250 nM budesonide and tacrolimus we had a concentration that was expected to be effective enough to cause a suppression without being toxic to the cells.

Activation of NF-κB represents a central event in the innate immune response of keratinocytes [3]. In our study 250 nM budesonide significantly inhibited the activation of NF-κB after treatment with LPS or TNF-α, while tacrolimus showed no effect. Although the LPS induction of NF-κB was not satisfactorily measurable in our assay, the slight induction observed was still inhibited by budesonide. These data correspond to the inhibition of both the constitutive as well as the LPS induced cytokine expression in keratinocytes by budesonide, but not by tacrolimus.

In our study we were not able to measure TNF-α protein in the supernatants, using two different TNF-α ELISA kits, even after LPS induction, although we detected a significant induction of TNF-α mRNA in the cells after LPS treatment. Some authors had similar difficulties with the detection of TNF-α in the supernatants of unstimulated cultured human keratinocytes by ELISA [57, 58]. Treatment with staphylococcal enterotoxin B, all-trans retinoic acid, phorbol-myristate-acetate and 10 mJ/cm^2 UVB resulted in the appearance of a small amount of TNF-α (6-25 pg/ml) detectable by ELISA in the supernatants of cultured keratinocytes [58, 59]. Pertussis toxin, diphtheria toxin or killed Staphylococcus species however did not show an effect on keratinocyte TNF-α production [57, 58]. At the same time Graham et al. observed TNF-α protein production both in control cells and keratinocytes stimulated with viable and nonviable Propionibacterium acnes [60].

The expression of TLR2 protein on keratinocytes has been shown by several investigators using immunohistochemistry [12, 61, 62]. The presence of TLR2 on the membrane of keratinocytes was also demonstrated by flow cytometry [63]. On the BEAS-2B transformed human bronchial epithelial cell line a strong increase in TLR2 surface expression has been shown by flow cytometry in TNF-α and IFN-γ stimulated cells after 1 μM dexamethasone treatment [64]. Furthermore, 1 μM dexamethasone synergistically enhanced the nontypeable Haemophilus influenzae-induced mRNA and protein expression of TLR2 on human cervix epithelial cell line (HeLa) via specific up-regulation of MAPK phosphatase-1 (MKP-1) that led to dephosphorylation and inactivation of p38 MAPK, the negative regulator for TLR2 expression [65, 66]. It has also been found that dexamethasone enhanced the IL-1β-induced TLR2 upregulation in the same cells using Western blot analysis [67]. In our study, budesonide showed similar effect on both unstimulated and LPS induced cultured human keratinocytes at the mRNA level generating a 3- to 5-fold TLR2 induction over the vehicle

treated samples. However, a mild, but significant stimulating effect of budesonide on the protein expression was only revealed in the LPS induced cells. It has been shown that glucocorticoids also act by posttranscriptional mechanisms. In human monocytes, dexamethasone was found to have a pronounced inhibitory effect on iNOS protein synthesis and decrease iNOS protein stability on rat renal mesangial cells [68]. Similar data have been reported for endotoxin-induced TNF-α expression in monocytes, where the inhibition of translation appeared to be the predominant mode of steroid action [69]. Steroids might influence the translation of TLR2 also and might contribute to the poor increase in TLR2 protein expression seen after budesonide treatment despite the strong increase in the TLR2 mRNA level.

We could detect only cytosolic TLR2 protein in our cultured keratinocytes. These cells represent the proliferating, undifferentiated cell population of keratinocytes. There is evidence that an increase in the cell surface TLR2 expression occurs in HaCaT keratinocytes in parallel with differentiation [18]. It is possible that in cultured keratinocytes the TLR2 membrane expression or the receptor transport to the cell surface is also restricted to the more differentiated cells.

Based on its general and wide-range suppressive effects on immune cells one would expect that steroids would inhibit most immune functions in keratinocytes as well. A well known side effect of steroids is the occurrence of steroid acne, frequently seen during systemic therapy. Gram-positive *Propionibacterium acnes* is likely to contribute to the characteristic inflammatory response in acne. As TLR2 can recognize Gram-positive bacterial cell wall components [1, 3, 12], it is possible that overexpression of TLR2 on keratinocytes after steroid treatment plays a role in the pathological inflammatory reaction to *P. acnes* seen in acne patients. On the other hand, topical corticosteroids may exacerbate cutaneous infections [70], which adverse effect might be partly due to the inhibitory effect of corticosteroids on the cytokine expression of keratinocytes suppressing the immune functions of cells.

In most inflammatory skin conditions cytokines released by keratinocytes participate in the development of skin inflammation. The fast occurring antiinflammatory effect that topically applied budesonide exerts on the skin could be partially due to its inhibition of the cytokine production by keratinocytes. The antiinflammatory action of tacrolimus takes a longer time to occur, maybe because it does not alter the cytokine production of keratinocytes. Our results show that tacrolimus and budesonide affects keratinocytes differently.

5.2 Effect of microbial compounds on IL-8, IL-1α and TNF-α protein expression in vaginal epithelial cell line

Vaginal epithelium has a powerful innate immune system that protects the female reproductive organs from bacterial and fungal infections. In our study, we aimed to explore whether the TLR signaling pathway and the induction of pro-inflammatory cytokines could contribute to the protection against pathogenic microorganisms in vaginal epithelial cells. IL-8 is one of the most potent neutrophil chemoattractants, which affects the ability of neutrophils to cross epithelial barriers and to kill bacteria as well [71, 72]. In our study, microbial compounds induced a strong, time-dependent activation of IL-8 gene in immortalized vaginal epithelial cells. Our results are in accordance with previous findings showing the activation of pro-inflammatory cytokines in vaginal epithelial cells after challenge with microbes and indicate that one of the most important immune functions of vaginal epithelial cells might be providing signals for inflammatory cells [30, 73].

We detected a significant induction of TNF-α mRNA in the cells after treatment with LPS, PGN and fungal compounds. However, the soluble TNF-α protein was not detectable in the supernatants of immortalized vaginal epithelial cells after treatment with microbial compounds, using ELISA kits purchased from two different manufacturers. We and other authors had similar difficulties with the detection of TNF-α in the supernatants of cultured human keratinocytes by ELISA (see above). TNF-α is initially synthesized as a 26-kDa pro-TNF-α, a type II integral membrane protein, and then is proteolytically cleaved to release a mature 17-kDa secreted form [74]. The cleavage of pro-TNF-α occurs primarily at the cell surface by specific enzymes [75]. Hence, the produced TNF-α protein may be either stored intracellularly and further microenvironmental stimuli are needed for its release to the extracellular matrix or it is bound to the cell surface, which could explain the virtual lack of this cytokine in the cell culture supernatants.

5.3 Examination of TLR2 and TLR4 polymorphisms in acne

Growing amounts of data suggest that the ability of certain individuals to respond properly to TLR ligands may be impaired by SNPs within TLR genes, resulting in an altered susceptibility to, or course of, infectious or inflammatory disease [41]. Association was found

between the occurrence of the two cosegregating polymorphisms of TLR4 (Asp299Gly and Thr399Ile) and the hyporesponsiveness to inhaled LPS. Investigators also found a clear correlation between severe respiratory syncytial virus bronchiolitis in infants and both TLR4 SNPs. Supporting the role of TLR2 in atopic diseases, the TLR2 Arg753Gln polymorphism was shown to define a subgroup of people with atopic dermatitis displaying a severe phenotype [41]. Our recent data have shown that certain *P. acnes* strains are capable to activate the innate immune responses in keratinocytes, and this induction is TLR2 and TLR4 dependent [76]. Beside keratinocytes, sebocytes and professional immune cells, e.g. macrophages, also express TLRs, through which *P. acnes* can contribute to inflammation at the site of acne lesions [40, 77]. Although the polymorphisms and mutations of TLR2 and TLR4 genes could be hypothesized to contribute to the pathogenesis of acne, the SNPs studied here were not proved to assist in the development of acne. The fact that we could not find any associations between acne and the studied TLR2 and TLR4 polymorphisms does not exclude the possibility that other polymorphisms of these two genes would contribute to acne pathogenesis. Further studies are needed to decide whether additional TLR2 and TLR4 polymorphisms are associated with acne and/or whether the reported upregulation of these genes in acne [39] is the result of upstream regulatory events.

We couldn't detect the TLR2 SNPs causing the amino acid changes Arg677Trp and Arg753Gln. This result corresponds to the data in the literature showing that Arg677Trp change does not occur and Arg753Gln shows a low allele frequency. According to our analysis by two computer programs, these two SNPs, especially the Arg677Trp change, have a considerable effect on the protein function and hydrophilicity. Presuming that an alteration in the protein function and hydrophilicity may have an impact on the occurrence of an SNP, the predicted effect of Arg677Trp and Arg753Gln change may explain why these two SNPs are so rare.

6. Summary

Epidermal cells play a role in several immune processes [3, 5]. In our study we intended to gain additional data on the innate immune functions of epidermal cells. We have investigated the effects of budesonide and tacrolimus on the IL-1α, TNF-α and IL-8 production and TLR2 and TLR4 expression in HaCaT cell line and in cultured keratinocytes isolated from normal human skin. Applying at the same concentration, the effect of these two immunosuppressants was different on IL-8, TNF-α, TLR2 and TLR4 genes in keratinocytes. This difference might explain the dissimilarity in the effects and side-effects of these two drugs after topical application on the skin. Our results, together with earlier data, confirm the cell-surface expression of TLR2 and TLR4 in keratinocytes and vaginal epithelial cells, through which these cells are capable of recognizing various kinds of microbial molecular patterns. Playing a role in the innate immune functions of the epidermis, these cells can contribute to the pathogenesis of several skin diseases. Data from the literature suggest, that TLRs participate in the development of acne [39, 40], hence the mutations and polymorphisms of these genes might have an influence on acne susceptibility. Although our study could not find any associations between acne and the studied TLR2 and TLR4 polymorphisms, it does not exclude the possibility that other polymorphisms of these genes would contribute to acne pathogenesis.

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Acknowledgements

I would like to thank Dr. Gyöngyvér Soós for her support and for providing me the opportunity to accomplish my scientific work.

I thank Prof. Dr. Lajos Kemény and Prof. Dr. Attila Dobozy for providing me the excellent opportunity to work at the Department of Dermatology and Allergology.

I am grateful to Dr. Zsuzsanna Bata-Csörgő, Dr. Márta Széll and Prof. Dr. Lajos Kemény for giving me invaluable advice, continuous support and encouragement.

I am also very grateful to Dr. Andrea Koreck, Hilda Polyánka, Dr. László Bodai, Dr. Andor Pivarcsi, Dr. István Nagy (Department of Dermatology and Allergology) and to Katalin Éder (Department of Biochemistry, Biological Research Center) and to all the colleagues, who provided me a lot of help in my work.

My special thanks to Prof. Dr. Ernő Duda from the Department of Biochemistry, Biological Research Center and to all the colleagues from Department of Immunology and Department of Parasitology, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania for the scientific co-operation.

I am grateful to Andrea Tanácsné Bajkán, Mónika Kohajda and Erzsébet Kusz for their skilled technical assistance.

APPENDIX